Investigating the recruitment of MSC from whole blood flow

by

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Abstract:

Mesenchymal stem cells (MSCs) are a population of adult stem cells located in the bone marrow. They are able to differentiate into cartilage, muscle, bone and fat. MSCs are an attractive therapeutic treatment option as they have been shown to be immunosuppressive and can be isolated and then expanded in culture. However delivering the MSCs to a site of damage or disease is not ideal as currently the best method is direct injection into the site which is highly invasive. Delivering the MSCs by injection into the circulation and then recruited to the damaged or diseased site would be a much safer and less invasive option. The aim of this study was to gain an insight into the ability of MSCs to be recruited to sites of damage or inflammation from the circulation by using an *in vitro* whole blood flow system. This study showed that MSCs were not able to become recruited from whole blood flow in this system when using shear rates equivalent to human circulation.

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1. INTRODUCTION

1.1 Mesenchymal stem cells

Mesenchymal stem cells (MSC) are a population of adult multipotent cells located in the bone marrow. MSCs have the ability to self renew *in vitro* and *in vivo*, and they can also differentiate into a number of different cell types including cartilage, muscle, bone and fat (Caplan, 1991, Prockop, 1997, Pittenger *et al.*, 1999). Characterization of MSCs is difficult as there are no known specific markers. MSCs are defined by their adherence to plastic, expression of surface markers CD90, CD73 and CD105, whilst being negative in expression of surface markers CD34, CD45, CD11b or CD14, CD19 or CD79α and HLA-DR; they must also possess the ability to differentiate into chondroblasts, adipocytes and osteoblasts (Dominici *et al.*, 2006). MSCs have a great therapeutic potential as they can be isolated and expanded in culture allowing autologous transplantation (Pittenger *et al.*, 1999). MSCs have also been shown to have an immunosuppressive effect (Di Nicola *et al.*, 2002) and are considered to be non-immunogenic (Klyushnenkova *et al.*, 1998, Bartholomew *et al.*, 2002). This opens up the potential to use allogenic transplantation if the immunosuppressive effect is enough to block the host immune system.

1.2 Animal models and clinical trials

Some research has been done to assess the utility of MSC transplantation. It has been shown that MSCs transplanted directly into a heart were able to differentiate into cardiomyocytes and improve myocardial function in animal models (Tomita *et al.*, 1999, Toma *et al.*, 2002). Tomita *et al*. (1999) showed that rat MSCs injected into a three week old cryoinjury-derived scar in the rat heart were able to differentiate into cardiac cells, improve

local angiogenesis and improve heart function. Toma *et al.* (2002) injected human MSCs into a healthy mouse heart and observed that a small population of the cells differentiated into cardiomyocytes. The success in mice has led to trials in human patients, which showed significantly improved left ventricular function in patients who had suffered acute myocardial infarction were treated with intracoronary injection of autologous MSCs (Chen *et al.*, 2004). Many other trials have been conducted using bone marrow mononuclear cells (BMMNC) with a range of results observed (Janssens *et al.*, 2006, Lunde *et al.*, 2006, Penicka, 2007). These animal studies and human clinical trials all used intraventricular injections of the MSCs/BMMNCs, which is a highly invasive technique that could potentially increase the risk of complications. A much safer method of administration would be intravenous injection but this would only be viable if the MSCs/BMMNCs had the ability to recognise damaged areas and adhere to them in sufficient quantities. Systemic intravenous injection of MSCs has been tested in *in vivo* rat models of myocardial infarction. The results showed that most of the transplanted cells became trapped in the lungs due to the large size of the MSCs compared to the relatively small size of the alveolar capillaries. However some were also located in the heart, spleen and liver (Barbash *et al.*, 2003). Other groups have had some success with intravenous injections of MSCs to treat cerebral ischemia (Chen *et al.*, 2001) and transplant rejection (Wu *et al.*, 2003)in rats. Another group has shown that bone marrow derived MSC transplant was able to improve bone mineral levels and dense bone formation in patients with osteogenesis imperfecta (Horwitz *et al.*, 1999). These results all show that MSCs have some potential to home to damaged tissues; the mechanisms for this homing are currently poorly understood but have been suggested to be analogous to leukocyte extravasation.

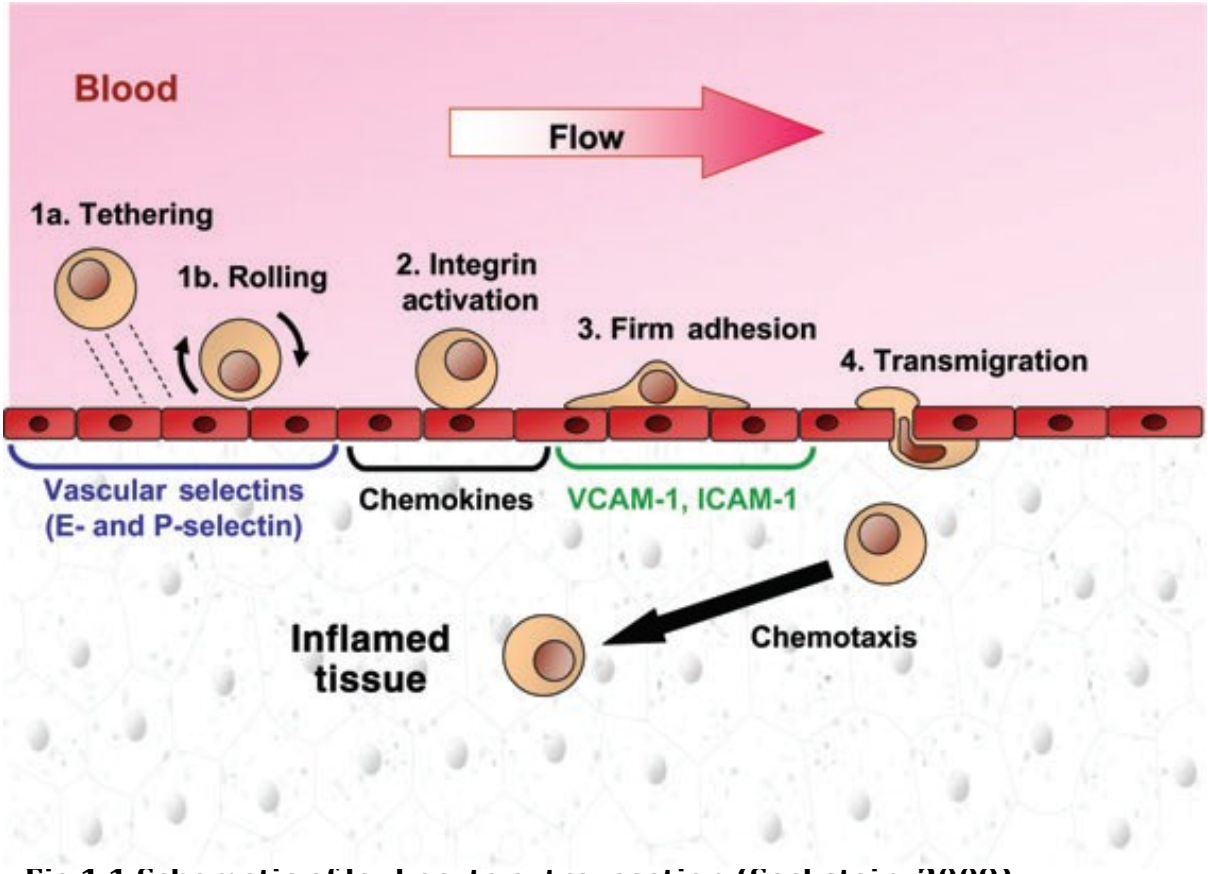

Fig 1.1 Schematic of leukocyte extravasation (Sackstein, 2009)

1.3 Leukocyte extravastaion

Leukocyte extravasation (fig 1.1) is a well documented process (Springer, 1995, Sackstein, 2009). Briefly; Leukocytes are able to respond to signals of local infection mediated by endothelial cells. The traffic signals involved work in a sequential manner beginning with the endothelial cell surface expression of selectins and ligands for leukocyte integrins, dependant on the type of endothelial cell and the cellular stimulus. The selectins will bind to molecules on the leukocyte surface weakly, slowing them down and bringing the leukocyte into closer contact with the endothelium. Next chemokines being released or presented by the endothelium will be able to have a direct effect on the leukocyte through specific receptors, promoting the activation of integrins on the surface of the leukocytes.

The activation of integrins allows the leukocytes to firmly adhere to the endothelium via interaction with immunoglobulin super-family members. The leukocyte can then migrate and penetrate the endothelium and move into the tissue.

1.4 Adhesion molecules on MSCs

MSCs have been shown to express several adhesion molecules which may be involved in their ability to bind to endothelium in order to pass through into tissue and aid in repair (De Ugarte *et al.*, 2003). Elucidation of mechanisms of adhesion may lead to the ability to manipulate MSCs to increase homing towards a specific site. MSCs have been observed to be able to adhere to endothelium in a flow model *in vitro* in a similar way to leukocytes, through binding to VCAM-1 and P-selectin (Ruester *et al.*, 2006). Manipulation of the CD44 on the surface of human MSCs through glycosyltransferase programmed stereo substitution to create a ligand for E-selectin was shown to increase binding of MSCs to endothelium (Thankamony & Sackstein, 2011). This kind of engineering could be used to increase site specific homing of MSCs. These groups studied the adherence and rolling of MSCs in suspension on their own, but the effects of the rest of the blood constituents which would be present if the MSCs were injected have not yet been studied. Furthermore the MSCs were flown into the assay at a very slow shear rate and allowed to adhere to the surface before the shear rate was increased to roughly levels found in the circulation of a human. It is therefore difficult to compare this model to the physiological system.

1.4 Chemokines and MSCs

MSCs have also been shown to express some chemokine receptors (Chamberlain *et al.*, 2007). Chemokines are an important subset of chemotactic cytokines with an important role in leukocyte trafficking and could possibly have an effect on the ability of MSCs to migrate

through endothelium. Circulating MSCs would need to be in close contact with endothelium in order for chemokines to be effective. Chemokine receptors on the MSCs could be used to induce controlled changes of expression in the MSCs potentially aiding the recruitment of the cells into the endothelium therapeutically. Segers *et al.* (2006) observed that pre-treating MSCs with TNF-α or IL-1β increased adherence to cardiac micro vascular endothelium (Segers *et al.*, 2006).

1.5 MSCs recruitment

The ability of MSCs to be recruited from the circulation in the body is not known. Leukocytes in whole blood are marginated to the walls of vessels due to the aggregation of red blood cells (Pearson & Lipowsky, 2000). The formation of red cell aggregates produces larger particles which flow at the centre of the vessel. The smaller leukocytes are then pushed outward towards the edge of the vessel; this mechanism allows the leukocytes to be in contact with the walls of the vessel and aid in leukocyte extravasation. In general, margination is more effective the slower the blood flow. It is possible that MSCs injected into the blood stream may also be marginated. However, MSCs are considerably larger than leukocytes and therefore may not be marginated as efficiently. There have been no studies of MSC margination to date.

Here, a previously established *in vitro* model of a glass capillary coated with adhesion molecules and using whole blood will be used to study the ability for MSCs to become captured by receptors which *in vivo* assist in the capture phase of leukocytes and platelets. Margination will also be assessed.

1.6 Hypothesis and aims

The Hypothesis of this project is that through margination and platelet bridges MSCs exhibit behaviour comparable to leukocytes at the attachment stage during extravasation.

The margination and adhesive behaviour of MSCs in blood is currently unknown; the aim of this project is to assess the ability of MSCs to bind to chosen adhesion molecules using an *in vitro* flow system to represent inflamed and damaged endothelium. Furthermore the interactions between MSCs and platelets will be assessed using flow cytometric analysis to identify potential MSC-platelet aggregates which may be involved in the recruitment process.

2. MATERIALS AND METHODS

2.1 Blood collection

Blood was taken from consenting healthy adult volunteers. Blood was mixed 9:1 with citrate phosphate dextrose adenine (CPDA; Sigma) anticoagulant.

2.2 Cell culture and growth conditions

Human mesenchymal stem cells (hMSC) were purchased from Lonza and grown in Mesenchymal Stem Cell Basal Medium (MSCBM) (Lonza) supplemented with Mesenchymal Cell Growth Supplement (MCGS), L-glutamine, and GA-1000 (Lonza). Cells were grown at a density of 2.5-3.5 x 10^5 cells per ml at 37°C in a humidified 5% CO_2-atmosphere. Medium was changed every 3-4 days. Cells were passaged at ~90% confluence. To remove the cells from the flask cells were washed with calcium and magnesium free phosphate buffered saline (PBS) solution. The PBS was removed from the flasks and 1 or 2ml EDTA was added to a 25cm^3 or a 75cm^3 flask respectively. Flasks were incubated at room temperature for 1 minute before adding 1 or 2ml Trypsin. Flasks were then incubated at 37°C for up to 5 minutes. Cells were observed under the microscope to confirm detachment from the plastic. Once detached 1 or 2ml of supplemented MSCBM was added to the flasks and the cell mixture was collected and centrifuged at 1100rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1ml MSCBM. 20µl was added to 20ml ISOTON® II Diluent (Beckman Coulter). The number and diameter of cells was determined using a Coulter Counter (Beckman Coulter). 1.5 x 10^6 cells per flow assay were removed from the cell suspension and the remaining cells were diluted to 2.5 x 10^5 cells per ml and added back into 75cm^3 culture flasks.

2.3 Flow system and adhesion assay

Glass capillaries with 50mm length, 3mm width and 0.3mm height were used in the flow system. The glass capillaries will be referred to as microslides.

3-aminopropyltriethoxysilane (APES) (Sigma) was used to coat the microslides to provide a surface for the proteins to bind. The microslides were washed overnight in 50% nitric acid, and then washed with running tap water for 4 hours. Excess water was removed using a suction pump and the microslides were allowed to dry. Microslides were then immersed in anhydrous acetone for 30 seconds whilst being repeatedly inverted. This process was repeated once and the microslides were dried by blotting on a paper towel. The microslides were then immersed in 4% APES in anhydrous acetone for 60 seconds whilst being repeatedly inverted and then dried by blotting. This process was repeated once and then the microslides were washed in anhydrous acetone. The microslides were washed three times with sterile distilled water using a pump. After the microslides had dried they were autoclaved at 121°C for 11 minutes.

Microslides were then coated with a variety of substrates which had been previously titrated for optimum concentration for leukocyte capture. Purified recombinant human P-selectin (R&D Systems) at 10µg/ml dissolved in PBS, Horm collagen (Axis-Sheild) at 500µg/ml, purified recombinant human VCAM-1 (R&D Systems) at 10µg/ml or a combination of 50% fibronectin (Sigma) at 10µg/ml and 50% p-selectin 10µg/ml. 50µl of each substrate or 50µl PBS as a negative control was pipetted into an individual microslide, and then the microslide was incubated at 37°C for one hour. After the 1 hour incubation, 150µl of 2% bovine serum albumin (BSA) in PBS was pipetted through each microslide to block non-specific protein-binding sites. The microslides were incubated at 4°C overnight.

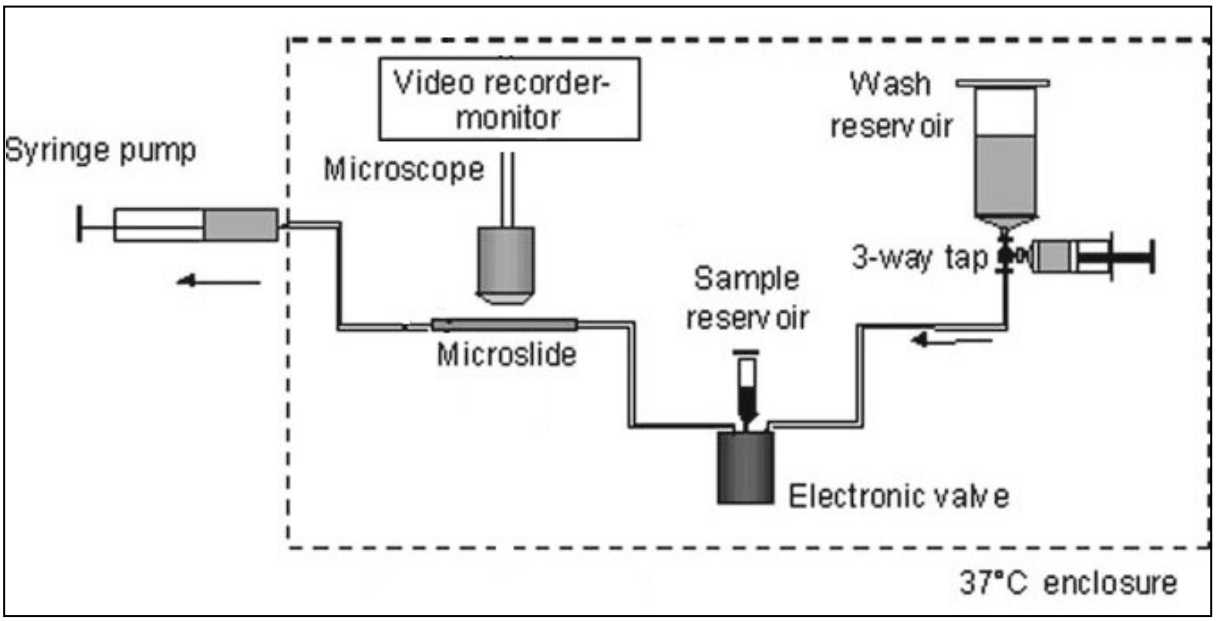

Fig 2.1 Schematic representing the flow system

The flow system was set up as in fig 2.1. The coated microslide was glued to the centre of a microscope slide and double-sided sticky tape adhesive was wrapped around the ends of the microslide. Each end of the microslide was then inserted into the rubber tubing and then placed under the microscope. With the electronic valve in the off position PBS without calcium and magnesium was flown through the system from the reservoir to the extraction syringe. 1ml of PBS without calcium and magnesium was added to the sample reservoir and the electronic valve was switched to the on position. The PBS in the sample reservoir was flown until a small amount remained in the neck of the syringe.

MSCs were added at a concentration of 1.5 x 10^5 per ml of whole blood in CPDA anticoagulant. MSCs were previously stained with CFSE (5µM, Molecular probes) fluorescent dye in order to allow visualisation on the fluorescence microscope. 5mM $MgCl_2$ or 1mM MnCl was added to the samples when desired, to enable integrin binding or induce integrin

activation respectively. In absence of added $MgCl_2$, CPDA anticoagulant (which chelates Ca^{++} and Mg^{++}) leaves enough residual Ca^{++} and Mg^{++} for selectin-mediated adhesion and platelet adhesion to collagen to be effective (Abbitt & Nash, 2001). However, leukocyte integrins at least require additional Mg^{++} to be effective. Mn^{++} directly induces the adhesive conformation of integrins without requirement for cell-activating stimuli. The syringe pump was set to deliver flow rates which delivered wall shear rates of 28 or $70s^{-1}$; the electronic valve was switched to the on position to allow the sample to flow through the microslide. The sample was perfused over the microslide for 2 minutes before events at the surfaces were recorded for 1 minute using a CCTV camera linked to a time-lapse video recorder. During the 1 minute recording the field of view was changed every 10 seconds. For flow cytometry experiments the perfused blood sample was collected from the three way valve at the end of the assay when using a BSA-coated microslide only.

The video was recorded at 50 frames per second and the width of the visible area was 0.75mm. This means that the speed of the passing MSCs can be determined by multiplying the frames it took to cross the screen by the frames per second and then dividing this by the size of the visible area. This calculation gives the speed of the passing MSCs in µm/second.

The percentage of all perfused MSC that were visible near the wall was calculated by counting the number of cells crossing the midpoint of the visible area in one second. This was then used to calculate the average number of cells crossing that point for the whole width of the microslide. The length of the midpoint of the screen was 0.59mm and the width of the microslide was 3mm. The syringe pump set at gear 8 withdraws 0.191ml volume per minute; therefore the 1ml blood sample containing $1.5x10^5$ MSCs will be flown through the flow assay in 314 seconds. This means that an average of 479 MSCs flow

through midpoint of the assay every second. With the pump set to gear 9 the withdrawal volume per minute was 0.0764ml. Therefore at this speed the average MSCs flowing through the midpoint of the assay every second was 191. The percentage of visible cells was then calculated at each wall shear rate to represent the amount of cells near the wall.

Wall shear rate = $\frac{6.Q}{w.h^2}$ where w = width of the microslide and h =height of the microslide and Q = volumetric flow rate.

Q = Pressure gradient x $\frac{wh^3}{12\eta}$ where w = width of the microslide and h =height of the microslide and η = blood viscosity.

2.4 Measurement of Haematocrit

Haematocrit was measured using a micro-haematocrit centrifuge. Whole blood in CPDA anticoagulant was drawn into a capillary tube through capillary action, the end of the tube was then blocked using Crystaseal and the tube was inserted into the micro-haematocrit centrifuge. The sample was centrifuged at 10,000rpm for 5 minutes. The haematocrit was calculated by measuring the height of the red cells in comparison to the whole sample. This was then converted into a percentage.

2.5 Flow cytometry

Samples collected from the flow assay plus a sample of blood without MSCs was diluted 1:10 with cold PBS plus 2% bovine serum albumin (PBSA). A sample of pure MSCs and the 1:10 dilutions were added to FACS tubes and centrifuged at 1500rpm, 4°C for 5 minutes.

Each pellet was resuspended in 100µl of directly conjugated antibody or 2% PBSA, (see table 2.1). The samples were incubated in the dark on ice for 30 minutes. 3ml cold 2% PBSA was added to each sample and then centrifuged at 1500rpm, 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 2ml 1:10 FACS lysing solution (BD Biosciences) and incubated for 10 minutes at room temperature. The samples were centrifuged at 1500rpm, 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 3ml 0.5% PBSA. The samples were centrifuged at 1500rpm, 4°C for 5 minutes. The supernatant was removed and the pellet was resuspended in 300µl 0.5% PBSA. The samples were analysed on DakoCytomation CyAn flow cytometer (Beckman Coulter).

Table 2.1 of antibodies used in flow cytometry

Antibody target	Conjugate	Rationale	Supplier	Dilution
CD105	FITC	Surface MSC marker	Ancell	1:50
CD42b	PE-Cy5	Surface Platelet marker	BD biosciences	1:5

2.6 Statistical analysis

Results were tested for statistical significance using a two factor analysis of variance (ANOVA) test.

3. RESULTS

3.1 Analysis of adhesion and margination of MSC in CPDA blood

The anticoagulant of choice, CPDA was used because it chelates calcium ions but leaves enough free to allow selectin mediated binding of leukocytes (Geng *et al.*, 1992). EDTA would chelate all of the calcium and therefore completely block any selectin or integrin binding. As it was hypothesised that MSC use a recruitment mechanism analogous to leukocyte extravasation; initially the same anticoagulant was used as in leukocyte adhesion studies with whole blood (Abbitt & Nash, 2001).

P-selectin was selected as the first microslide coating because P-selectin is known to be an important ligand which aids in the tethering and rolling of leukocytes. P-selectin is up regulated on endothelial cell surface during inflammation. It is possible that MSCs may express a receptor which recognises p-selectin and may also roll in a similar mechanism to leukocytes. Ruester *et al.* (2006) showed that blocking p-selectin on HUVEC cells lowered the number of MSCs which firmly adhered after being perfused (Ruester *et al.*, 2006). This suggests that MSCs may be able to bind to p-selectin.

Collagen was used as a model ligand for an area of vessel damage as collagen is exposed when tissue is damaged with exposure of the basement membrane. Collagen also activates platelets and platelets readily bind to it (Moroi *et al.*, 1996). The rationale behind using collagen was that the platelets may bind to the MSCs and facilitate MSC binding onto the collagen or that the platelets which bind to the collagen may provide a region for an MSC to adhere to (as is the case for leukocytes) or that a combination of both might occur. There was also a possibility that the MSCs might bind directly to the collagen.

1% PBSA was used to block non-specific binding after the collagen and p-selectin were allowed to bind to the microslide. The same 1% PBSA was used to coat a microslide which had not been coated with collagen or p-selectin. This was used as the negative control slide in this set of experiments.

Initially a flow assay with rhodamine 6g labelled CPDA anticoagulated whole blood was observed, using P-selectin and Horm collagen coated microslides, to compare to any adhesion by the MSCs. Leukocytes were observed to roll on the p-selectin and platelets were observed to firmly adhere on the collagen (data not shown).

The flow assay with MSCs was carried out using the same microslide coatings, however the MSCs were not observed to stably adhere to either surface coating. Therefore to assess if the surfaces were having any effect on the MSCs the average velocity of the MSCs was calculated. Minor changes in the velocity of the MSCs could be attributed to the MSCs attaching to the surfaces but not beginning to roll.

Fig3.1 shows the average velocities of the MSC flowing across albumin, P-selectin or collagen at two different wall shear rates. The coating of the microslide did not have a statistically significant effect on the average velocity of the MSCs however as expected the change in wall shear rate did statistically significantly affect the velocity shown by ANOVA.

Fig3.2 shows the percentage of perfused MSC that were visible near the wall. While there tended to be a greater percentage of cells near the wall at the lower shear rate, ANOVA showed that the coating of the microslide and the shear rate had no significant effects on the percentage visible.

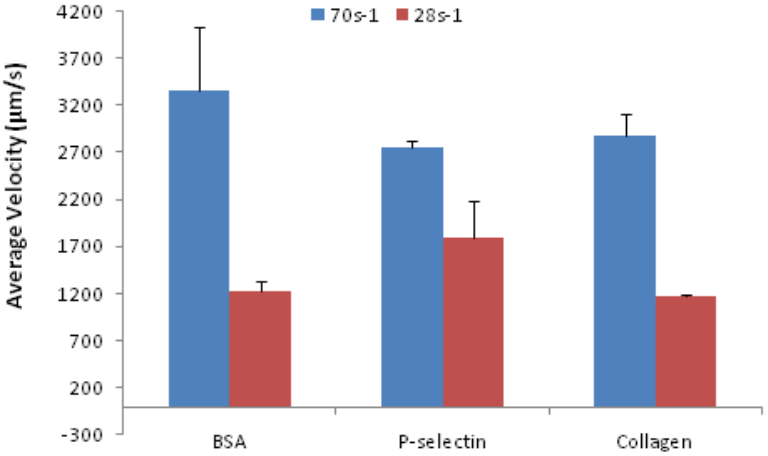

Fig 3.1 Effects of wall shear rate on velocity of MSC perfused over different adhesion substrates

1.5 x 10^5 CFSE labelled hMSC per ml whole blood, anti-coagulated with CPDA, were perfused through 300 x 3000µm microslides coated with P-selectin (10µg/ml), Horm collagen (500µg/ml), or BSA (1%) alone at 70s^{-1} or 28s^{-1} wall shear rate. Number of frames for MSC to cross the visible area were counted for 50 cells during the last minute of a 3 minute bolus. Velocities of MSCs were calculated based on 1 frame = 0.02s. Data are mean +/- SEM of the means from 3 experiments.

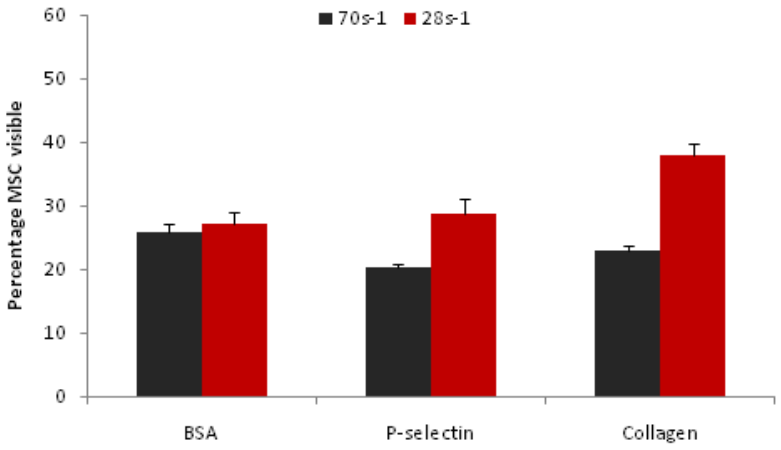

Fig 3.2 Effects of wall shear rate on surface visibility of MSC perfused over different adhesion substrates

1.5 x 10^5 CFSE labelled hMSC per ml whole blood, anti-coagulated with CPDA, were perfused through 300 x 3000µm microslides coated with P-selectin (10µg/ml), Horm collagen(500µg/ml), or BSA(1%) alone at 70s^{-1} or 28s^{-1} shear rate. Number of cells to cross the centre of the visible area were counted for 1 second during the last minute of a 3 minute bolus. Percentage of visible MSCs was calculated. Error bars represent. Data are mean +/- SEM of the means from 3 experiments.

3.2 Analysis of adhesion and margination of MSC in CPDA blood with MgCl2 added

In order to test whether there is a requirement for magnesium or calcium ions to be present to allow for integrins binding, 5mM $MgCl_2$ was added to each of the samples before it was put into the flow assay. The addition of $MgCl_2$ will also liberate calcium ions from the citrate of the anticoagulant which could enable other adhesion molecules to function more effectively.

This set of experiments used the same coatings as in the previous set, however in addition VCAM-1 and a combination of p-selectin and fibronectin were used. VCAM-1 has been suggested to be important in MSC adherence and MSCs have been shown to express VLA-4 which is the receptor for VCAM-1 (Ruester *et al.*, 2006).

Fig 3.3 shows the average velocities of the MSC flowing across albumin, P-selectin, collagen, VCAM-1, P-selectin and fibronectin, or BSA alone at two different wall shear rates with addition of $MgCl_2$. The coating of the microslide did not have a statistically significant effect on the average velocity of the MSCs however as expected the change in wall shear rate did statistically significantly affect the velocity shown by ANOVA.

Fig 3.4 shows the percentage of perfused MSC that were visible near the wall. While there appears to be a higher percentage of cells near the wall at the lower shear rate, ANOVA showed that the coating of the microslide and the shear rate had no significant effects on the percentage visible.

In some of the collagen coated microslide experiments small numbers MSCs were observed to be firmly adhered during the recording phase of the bolus. This result was not reproducible but was not observed with any of the other microslide coatings and was not observed in the absence of $MgCl_2$.

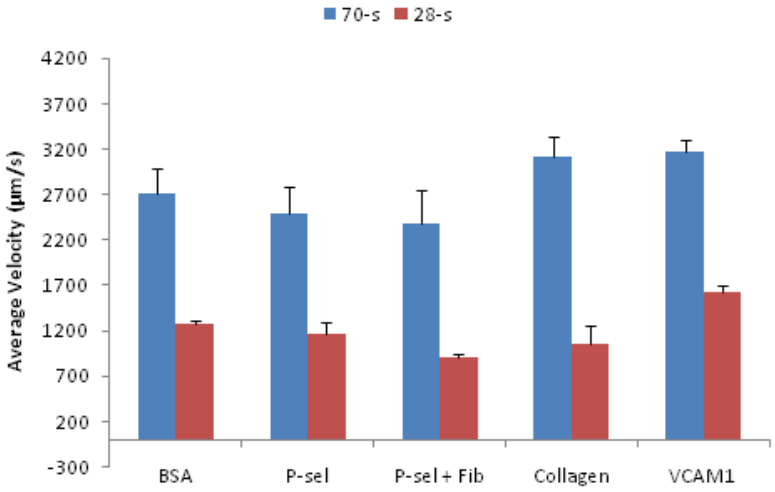

Fig 3.3 Effects of wall shear rate on velocity of MSC perfused over different adhesion substrates with $MgCl_2$

1.5 x 10^5 CFSE labelled hMSC per ml whole blood(+5mM $MgCl_2$), anti-coagulated with CPDA, were perfused through 300 x 3000µm microslides coated with P-selectin (10µg/ml), P-selectin and fibronectin (10µg/ml) Horm collagen(500µg/ml), VCAM-1(10µg/ml) or BSA(1%) alone at 70s^{-1} or 28s^{-1} shear rate. Numbers of frames to cross the visible area were counted for 50 cells during the last minute of a 3 minute bolus. Average velocity of MSCs was calculated based on 1 frame = 0.02s. Data are mean+/-SEM of the means from 3 experiments.

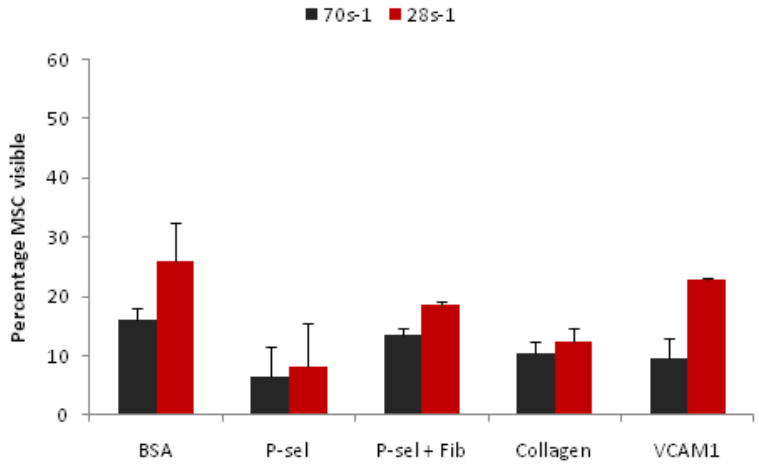

Fig 3.4 Effects of wall shear rate on surface visibility of MSC perfused over different adhesion substrates with $MgCl_2$

1.5 x 10^5 CFSE labelled hMSC per ml whole blood(+5mM MgCl2), anti-coagulated with CPDA, were perfused through 300 x 3000µm microslides coated with P-selectin (10µg/ml), P-selectin and fibronectin (10µg/ml) Horm collagen(500µg/ml), VCAM-1(10µg/ml) or BSA(1%) alone at 70s^{-1} or 28s^{-1} shear rate. Numbers of cells to cross the centre of the visible area were counted for 1 second during the last minute of a 3 minute bolus. Percentage of visible MSCs was calculated. Data are mean +/- SEM of the means from 3 experiments.

3.3 Analysis of adhesion and margination of MSC in CPDA blood with MnCl added

Collagen was selected to be followed up in the following experiments because of the previously mentioned occurrence of MSCs firmly adhered to the surface of some collagen coated microslides.

Manganese ions have previously been shown to non-specifically activate integrins at relatively low concentrations (Smith *et al.*, 1994). A previously established concentration of 1mM MnCl was added to each sample before it was perfused through the flow system.

Fig 3.5 shows the average velocities of the MSC flowing across collagen or VCAM-1 at two different wall shear rates with or without the addition of MnCl. The coating of the microslide, the addition of MnCl and the change in wall shear rate did not have a statistically significant effect on the average velocity of the MSCs shown by ANOVA.

Fig 3.6 shows the percentage of perfused MSC that were visible near the wall. While there tended to be a greater percentage of cells near the wall at the lower shear rate, ANOVA showed that the coating of the microslide, the addition of MnCl and the shear rate had no significant effects on the percentage visible.

Similarly to the $MgCl_2$ experiments, some MSCs were observed to firmly adhere on the collagen coated microslides in over 50% of the MnCl positive experiments. In contrast there were none observed in any of the MnCl negative experiments.

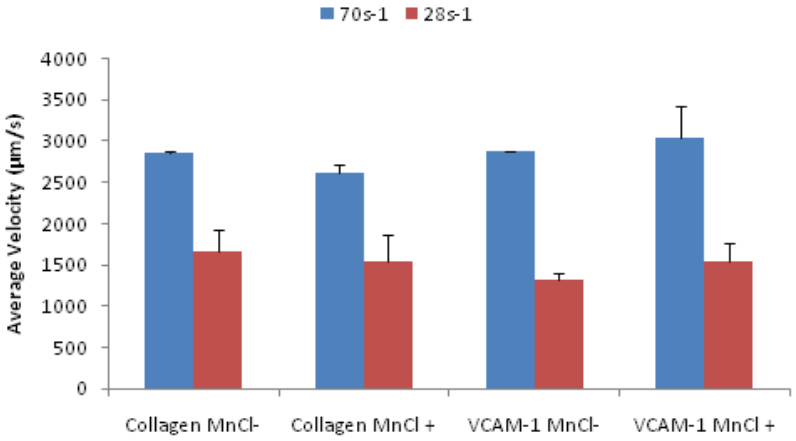

Fig 3.5 Effects of wall shear rate on velocity of MSC perfused over different adhesion substrates with or without MnCl

1.5 x 10^5 CFSE labelled hMSC per ml whole blood with or without 1mM MnCl, anti-coagulated with CPDA, were perfused through 300 x 3000µm microslides coated with Horm collagen (500µg/ml) or VCAM-1(10µg/ml) at 70 s^{-1} or 28 s^{-1} shear rate. Numbers of frames to cross the visible area were counted for 50 cells during the last minute of a 3 minute bolus. Average velocity of MSCs was calculated based on 1 frame = 0.02s. Data are mean +/- SEM of the means from 3 experiments.

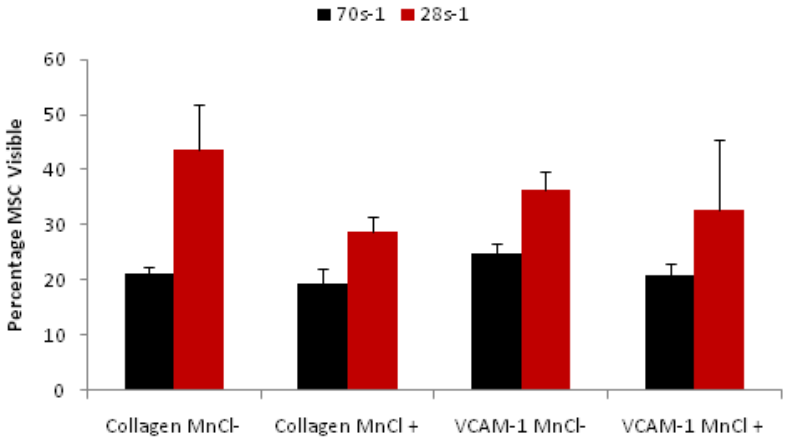

Fig 3.6 Effects of wall shear rate on surface visibility of MSC perfused over different adhesion substrates with or without MnCl

1.5 x 10^5 CFSE labelled hMSC per ml whole blood with or without 1mM MnCl, anti-coagulated with CPDA, were perfused through 300 x 3000µm microslides coated with Horm collagen (500µg/ml) or VCAM-1(10µg/ml) at 70 s^{-1} or 28 s^{-1} shear rate. Numbers of cells to cross the centre of the visible area were counted for 1 second during the last minute of a 3 minute bolus. Percentage of visible MSCs was calculated. Data are mean +/- SEM of the means from 3 experiments.

3.4 The effect of TNFα on MSC flowing velocity and margination

Stimulating MSCs with the cytokine TNFα for 24 hours may increase the surface expression of ligands which bind to VCAM-1, collagen and/or platelets. Although this stimulation is not likely *in vivo* it would be possible to stimulate autologous culture expanded human MSCs before transplantation. The stimulation could potentially improve the homing of the transplanted cells.

Fig 3.7 shows the average velocities of the MSC flowing across collagen or VCAM-1 at two different wall shear rates with or without 24 hour MSC TNFα treatment. Due to time constraints only one TNFα stimulation flow experiment was carried out. The TNFα stimulated MSCs perfused through VCAM-1 microslides were slower at both wall shear rates when compared to un-stimulated MSCs. This observation was also true for the MSCs perfused through the collagen microslide at $70s^{-1}$, however there was no effect observed in the collagen microslide at $28s^{-1}$. The statistical significance of these observations cannot be tested as there are too few replicates.

Fig 3.8 shows the shows the percentage of perfused MSC that were visible near the wall. Due to time constraints only one TNFα stimulation flow experiment was carried out.

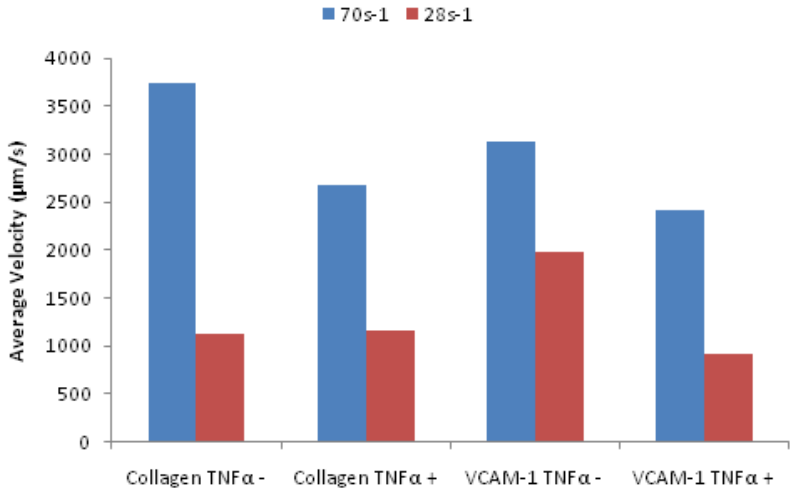

Fig 3.7 Effects of wall shear rate on velocity of MSC perfused over different adhesion substrates with or without TNFα treatment
1.5 x 10^5 CFSE labelled hMSC (stimulated or not stimulated with TNFα) per ml whole blood (+ 5mM $MgCl_2$), anti-coagulated with CPDA, were perfused through 300 x 3000µm microslides coated with Horm collagen (500µg/ml) or VCAM-1(10µg/ml) at 70 s^{-1} or 28 s^{-1} shear rate. Numbers of frames to cross the visible area were counted for 50 cells during the last minute of a 3 minute bolus. Average velocity of MSCs was calculated based on 1 frame = 0.02s. Data are the mean from one experiment.

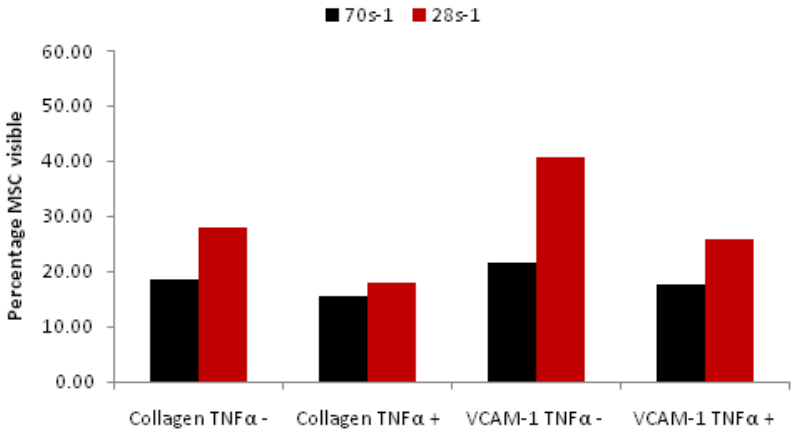

Fig 3.8 Effects of wall shear rate on surface visibility of MSC perfused over different adhesion substrates with or without TNFα treatment
1.5 x 10^5 CFSE labelled hMSC (stimulated or not stimulated with TNFα) per ml whole blood (+ 5mM $MgCl_2$), anti-coagulated with CPDA, were perfused through 300 x 3000µm microslides coated with Horm collagen (500µg/ml) or VCAM-1(10µg/ml) at 70 s^{-1} or 28 s^{-1} shear rate. Numbers of cells to cross the centre of the visible area were counted for 1 second during the last minute of a 3 minute bolus. Percentage of visible MSCs was calculated. Data are the mean from one experiment.

3.5 Analysis of platelet binding to MSCs in whole blood

The flow system described in section 2.3 was used but the MSCs were not stained with CFSE. MgCl2 or MnCl were added to the two of the samples before they were perfused through a 1% PBSA coated microslide. The sample was collected from the valve near the withdrawal syringe. A sample of blood without MSCs and a sample of pure MSCs was also retained.

Unstained MSCs were analysed for their side scatter, front scatter (fig 3.9 (A)), FITC and PE-Cy5 fluorescence (fig 3.9 (B, C & D)). The population of MSCs was observed to be quite diverse in size and granularity. The size variation was also observed when the size of the MSCs was analysed on the coulter counter. The levels of the FITC and PE-Cy5 were taken to determine the levels of auto-fluorescence of the MSCs.

MSCs stained with the CD105 FITC conjugated antibody were analysed in order to assess the quality of the staining process and to make sure that the FITC fluorophore is not giving an emission in the PE-Cy5 channel. Fig 3.10 shows that the FITC fluorescence was increased by a factor of 10 compared with the unstained MSCs. The PE-Cy5 fluorescence intensity remained the same as the unstained MSCs showing that the FITC labelling is not having an effect on the PE-Cy5 emission levels.

The sample without $MgCl_2$ or MnCl (fig 3.11) was labelled with the CD105 and CD42b antibodies. The first stage of the analysis was to gate for the MSC population (R1). The FITC fluorescence intensity increased by a factor of 10 compared to the unstained MSCs. The area of FITC intensity was then gated (R6) to identify changes in the PE-Cy5 fluorescence. The PE-Cy5 fluorescence intensity remained at the level of the unstained MSCs.

The sample with $MgCl_2$ was labelled with the CD105 and CD42b antibodies (fig 3.12). The same gating strategy as previously used was applied to this sample. This sample contained a number of cells which stained positive for both CD105 and CD42b antibodies, indicating some interaction between MSCs and platelets in the presence of $MgCl_2$.

The sample with MnCl was labelled with the CD105 and CD42b antibodies (fig 3.13). The same gating strategy as previously used was applied to this sample. As with the MgCl2 sample a population of cells was observed to be stained with both antibodies, showing an interaction between platelets and MSCs.

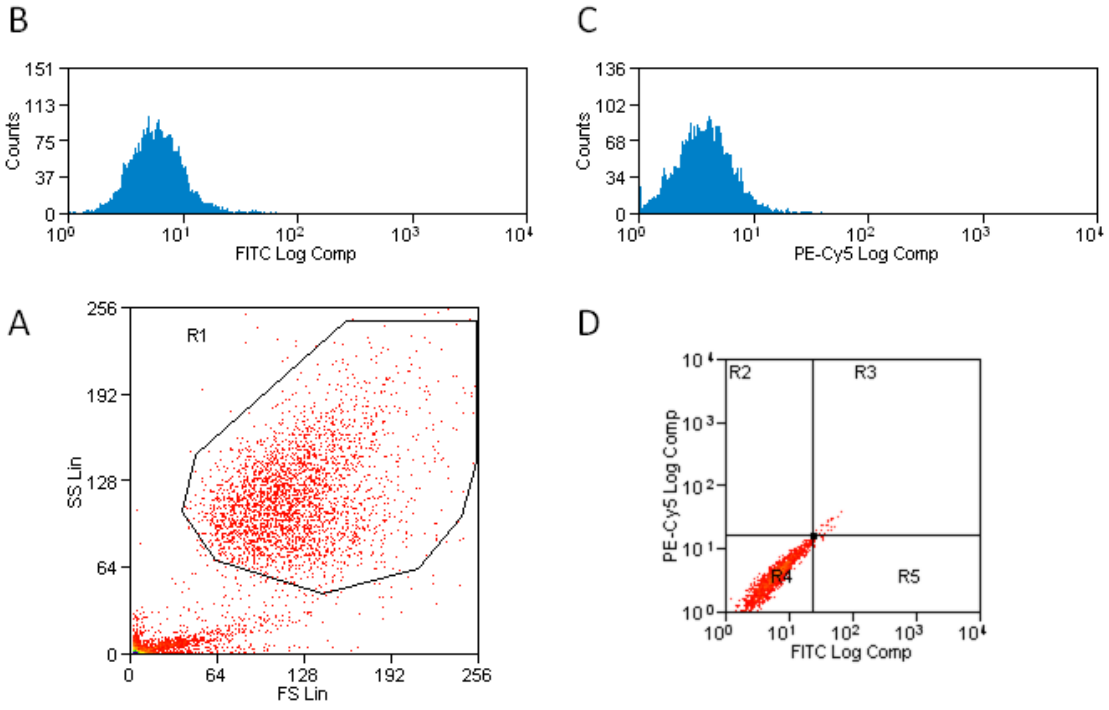

Fig 3.9 Flow cytometric analysis of unstained MSCs
A sample of unstained MSCs was analysed by flow cytometry. (A) Two dimensional dot plot showing live cells, R1 shows where cells were gated. (B & C) Histogram representing the fluorescence intensity of FITC and PE-Cy5 respectively. (D) Two-parameter histogram representing the fluorescence intensity of the FITC and PE-Cy5.

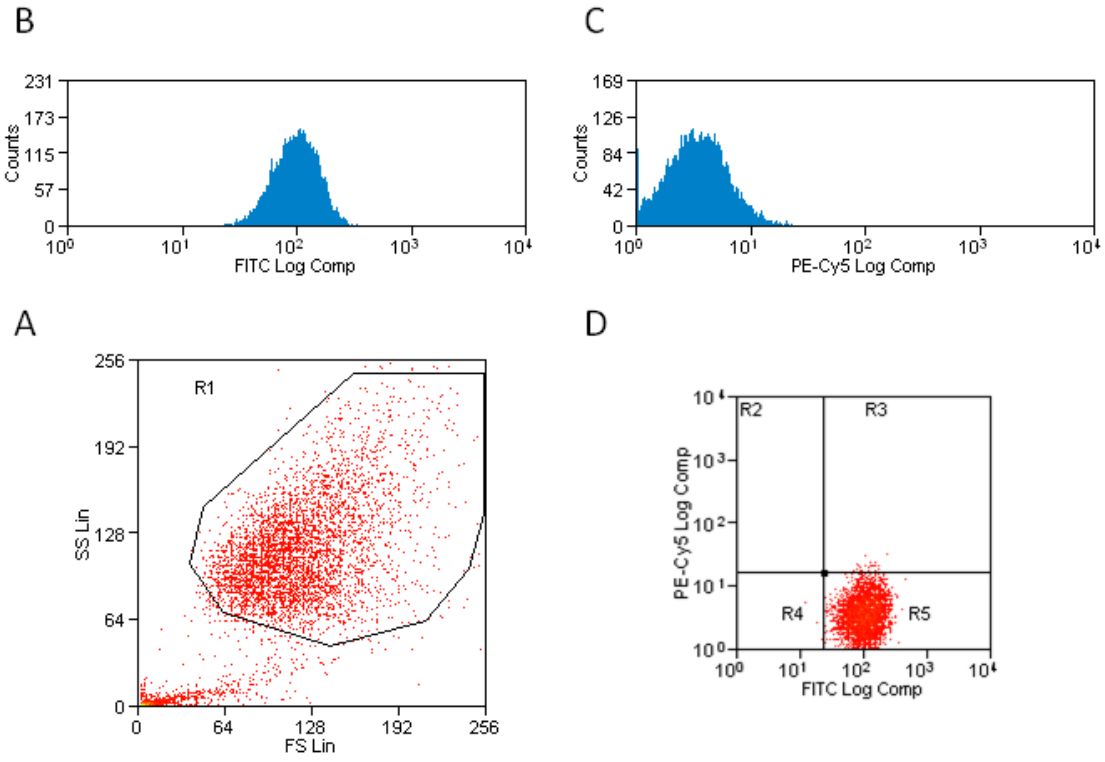

Fig 3.10 Flow cytometric analysis of anti-CD105 labelled MSCs
A sample of anti-CD105 conjugated with FITC fluorochrome stained MSCs was analysed. . (A) Two dimensional dot plot showing live cells, R1 shows where cells were gated. (B & C) Histogram representing the fluorescence intensity of FITC and PE-Cy5 respectively. (D) Two-parameter histogram representing the fluorescence intensity of the FITC and PE-Cy5.

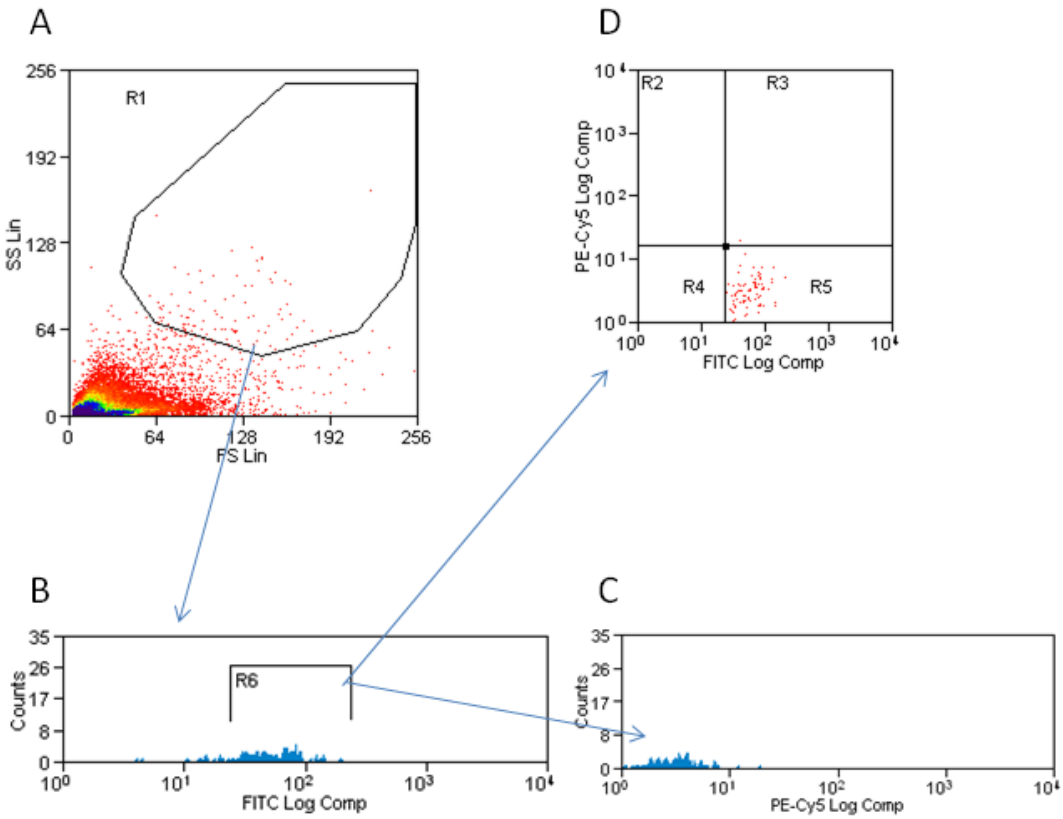

Fig 3.11 Flow cytometric analysis of anti-CD105 and anti-CD42b labelled flow system extract
A sample of blood after it passed through the flow system with MSCs. . The sample was stained with anti-CD105 antibody conjugated with FITC fluorochrome and anti-CD42b antibody conjugated with PE-Cy5 fluorochrome. Arrows represent the gating strategy. (A) Two dimensional dot plot showing live cells, R1 shows where cells were gated. (B & C) Histogram representing the fluorescence intensity of FITC and PE-Cy5 respectively. (D) Two-parameter histogram representing the fluorescence intensity of the FITC and PE-Cy5.

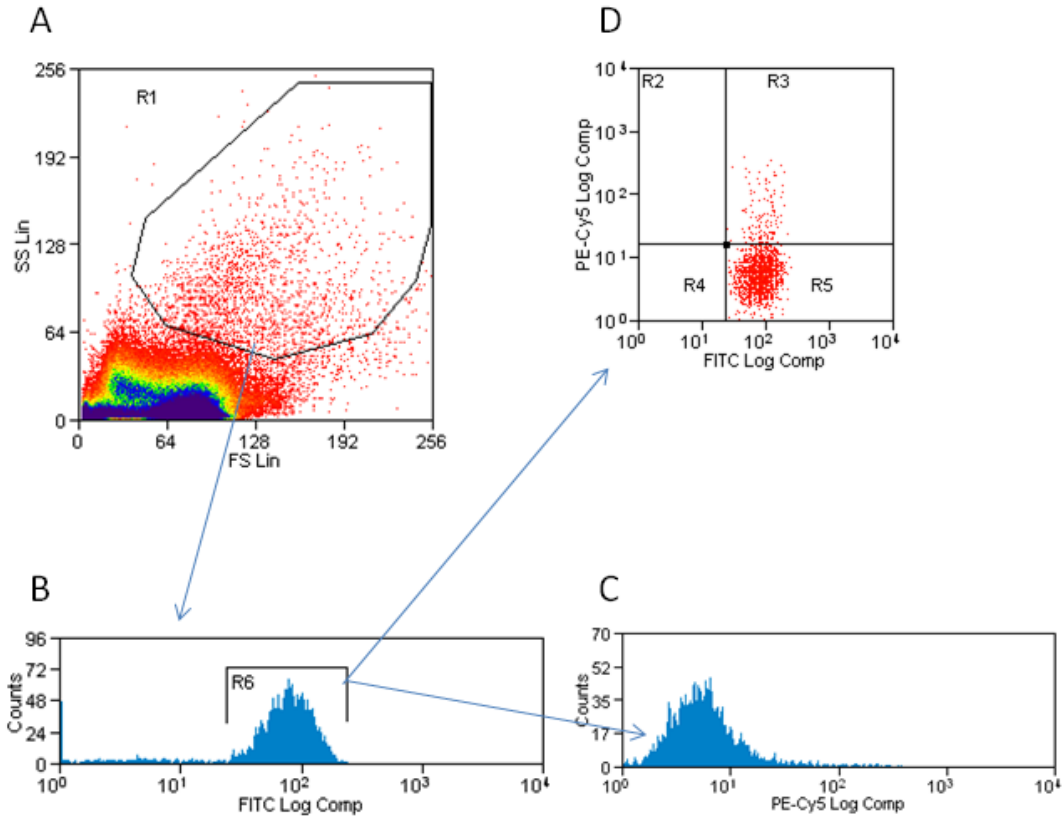

Fig 3.12 Flow cytometric analysis of anti-CD105 and anti-CD42b labelled flow system extract
A sample of blood after it passed through the flow system with MSCs and 5mM $MgCl_2$. . The sample was stained with anti-CD105 antibody conjugated with FITC fluorochrome and anti-CD42b antibody conjugated with PE-Cy5 fluorochrome. Arrows represent the gating strategy. (A) Two dimensional dot plot showing live cells, R1 shows where cells were gated. (B & C) Histogram representing the fluorescence intensity of FITC and PE-Cy5 respectively. (D) Two-parameter histogram representing the fluorescence intensity of the FITC and PE-Cy5.

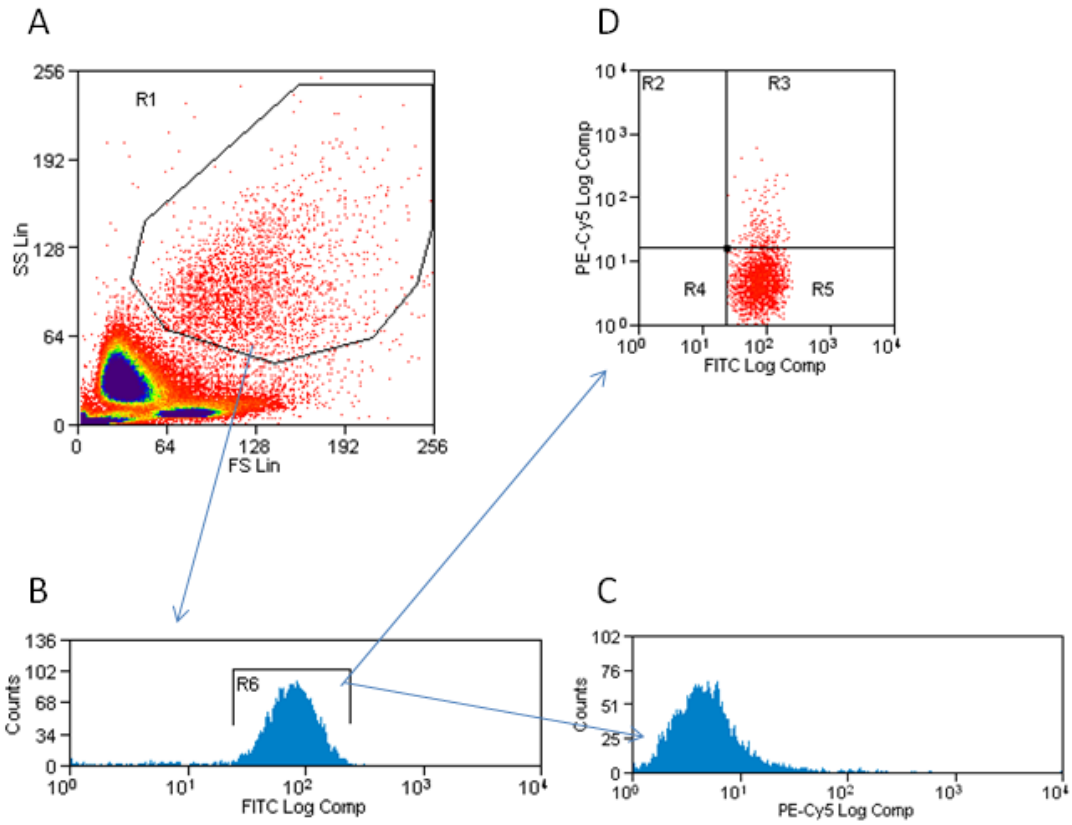

Fig 3.13 Flow cytometric analysis of anti-CD105 and anti-CD42b labelled flow system extract
A sample of blood after it passed through the flow system with MSCs and 1 mM MnC1. The sample was stained with anti-CD105 antibody conjugated with FITC fluorochrome and anti-CD42b antibody conjugated with PE-Cy5 fluorochrome. Arrows represent the gating strategy. (A) Two dimensional dot plot showing live cells, R1 shows where cells were gated. (B & C) Histogram representing the fluorescence intensity of FITC and PE-Cy5 respectively. (D) Two-parameter histogram representing the fluorescence intensity of the FITC and PE-Cy5.

4. DISCUSSION

The results presented in section3 suggest that un-stimulated MSCs are unable to be captured from flow in the whole blood flow assay even at very low wall shear rates. There are a number of factors which could contribute to the lack of adherence. Firstly there may not be enough surface receptors on the MSCs to bind to the P-selectin, collagen, VCAM-1 or fibronectin proteins. With too few receptors the MSCs will not be sufficiently slowed down to observe any change in their behaviour. The data in section 3 show that there was no significant variation in the speed of MSC when using the different protein coatings. After perfusion of blood, the coatings were tested by observing the washout using phase contrast optics. The P-selectin and fibronectin coatings were observed to have leukocytes rolling on the surface. Rolling and stationary leukocytes were observed on the VCAM-1 coated microslides and platelets were seen to aggregate and adhere to the collagen slides. These observations show that the coatings were adequate as the blood constituents behaved as expected.

For successful capture the bonds between the cell and the capture proteins must be strong enough to overcome the force of the prevailing blood, or at least enough to allow for a series of small interactions which would slow the cell down. The force on the cell is greater on a cell with a greater radius (Chang & Hammer, 1996). The median radius of the MSCs was observed to be 19-21µm which is considerably larger than circulating leukocytes (6-8µm (Downey *et al.*, 1990)) and platelets (1.5-3µm (Bain, 2006)). This size difference means that the MSC are under a much greater force than the leukocytes and platelets and therefore less likely to become captured by the adhesion proteins. Coupled with this is the fact that the due to their size, MSCs have a larger surface area which will mean that the density of

any receptors for the capture proteins is lower and the efficiency of capture will therefore be lower.

In normal blood flow red cells form aggregates which flow in the centre of the vessel, and these aggregates force the smaller platelets and leukocytes out of the centre of the vessel, increasing contact of platelets and leukocytes with the vessel wall. The size of the MSCs is so much larger than the leukocytes that the margination might be less effective. The results in section 3 for the numbers of MSC fllowing near the wall, show that the margination of the MSCs remained unchanged across the variety of microslide coatings (as would be expected) and was only significantly affected by changing the wall shear rate in one experiment. It has been shown that the aggregation of red blood cells increases as the wall shear rate drops (Goldsmith & Turitto, 1986), so that the MSCs should become more marginated at the 28s-1 shear rate. The results do show a slight increase in the margination at the lower wall shear rate, but this increase was not significant. If the MSCs are travelling down the centre of the vessel due to their large size their contact with the wall will be limited severely, and without contact the MSCs will not be able to adhere.

Adding 1mM MnCl is enough to non-specifically activate any integrins on the surface of the MSCs. However there was no observed decrease in the velocity of the MSCs in the samples with MnCl indicating that there was no increased adherence of the MSCs to the capture proteins tested. If there are too few integrins on the surface, even when activated the MSCs will not become captured due to the force on the cells as they try to bind to the capture proteins. The combination of a low number of integrins and the large force on the cells due to their size means the chances of capture are very low.

Ruester *et al.* (2006) and Thankamony *et al.* (2011) observed MSCs adhering and rolling in flow assays using MSCs in isolated suspension. In the whole blood assay tested in this project the binding and rolling of leukocytes and platelets could be hampering the ability of the larger MSCs to become captured. The leukocytes and platelets have a higher affinity for their respective capture proteins and therefore are likely to obstruct the MSCs.

The small number of MSCs observed to be firmly adhered to the collagen coated microslides could be due to platelets binding to the MSCs and then aiding in the capture to the collagen through the platelets von Willebrand factor (vWF). The results in section 3.5 show that there is platelet MSC interaction in the experimental flow assay. This result does not confirm whether the platelets bound to the MSCs aided in capture, or if the platelets bound to the collagen and then the MSCs were able to bind to the platelet aggregates. There is also a third option; the MSC could be binding directly to the collagen. Due to the low number of cells observed to adhere and the consequent variaility, this is difficult to test. However activating the platelets in the blood before they enter the flow assay might increase the number of MSCs firmly adhered. If the number of bound MSCs did not increase then this would be evidence that the MSCs were binding directly to the collagen. If the number of bound MSCs increased then the other two mechanisms are more likely. It is likely that both these mechanisms are at work, and it may be possible to elucidate which mechanism is more influential in the binding of the MSCs by blocking vWF, p-selectin or other adhesion molecules found on the platelets. By using the flow assay and then also analysing the perfusate by flow cytometry, it may be possible to identify the ligands involved in platelet-MSC adhesion. Platelets bind to exposed collagen *in vivo* to form a thrombus, and circulating MSCs could potentially adhere to thrombus in order to aid in repair of damaged tissue.

The results in section 3.4 represent only one experiment in which the MSCs were stimulated with the chemokine TNFα due to time constraints. This experiment should be repeated to determine if the effect of TNFα is significant. Other chemokines such as IL-1β could also be tested. With 24 hour stimulation it might be possible for the MSCs to change their transcription of adhesion molecules on their surfaces, and this might lead to increased rolling and potentially stable adherence. This level of cytokine stimulation is not likely *in vivo* but could be usefully exploited in an *ex vivo* transplant situation.

The anticoagulant could be changed as the chelating of the calcium ions may be too effective. An anticoagulant which does not alter the calcium ion concentration could be used as the MSCs may require more calcium ions to be present before they are able to begin to adhere.

Another consideration is the possible effect of using Trypsin to disassociate the MSCs from the culture flasks. The Trypsin could be cleaving adhesion molecules from the cell surface. If this was the case MSC might not have been able to re-express them before the flow assay was done. An enzyme free disassociation buffer could be used, EDTA or mechanical scraping of the cells. If time had allowed these would have all been tested in the flow assay to determine which method was the least damaging to the MSCs adhesion molecules.

In addition even though there was no observed rolling or adherence on P-selectin in these assays, there is a possibility that the other two members of the selectin family, E and L selectin, may be involved in recruitment of MSCs from the circulation. These two selectins could be assayed in the same experimental procedure.

In conclusion these data show that un-stimulated MSCs do not readily adhere or roll in whole blood at shear rates much lower than the majority of *in vivo* human circulation. There

is some interaction between MSCs and platelets but their relationship needs to be investigated further before any further conclusions can be drawn.

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